

Published in final edited form as:

*Mol Pharm.* 2011 February 7; 8(1): 297–301. doi:10.1021/mp100363f.

## Imbuing Aqueous Solubility to Amphotericin B and Nystatin with a Vitamin

Timothy P. Day<sup>1</sup>, Diptesh Sil<sup>1</sup>, Nikunj M. Shukla<sup>1</sup>, Asokan Anbanandam<sup>2</sup>, Victor W. Day<sup>3</sup>, and Sunil A. David<sup>1,\*</sup>

<sup>1</sup> Department of Medicinal Chemistry, University of Kansas

<sup>2</sup> Delbert M. Shankel Structural Biology Center, University of Kansas

<sup>3</sup> The Small-Molecule X-Ray Crystallography Laboratory, University of Kansas

### Abstract

Aqueous solubilities of many drugs in current clinical use are very low, necessitating formulations that often present problems for parenteral administration, including toxicities due to the excipients used. Recognizing that pharmacologically active compounds frequently possess amines, we asked whether pyridoxal phosphate (PLP), an innocuous, water-soluble vitamin, could be utilized to form prodrug-like complexes via the formation of imine or iminium adducts, and whether the vitamin would impart solubilizing properties to such complexes. Direct spectroscopic and crystallographic data obtained using model primary and secondary amines showed that PLP forms stable imine adducts with primary amines under entirely aqueous conditions and at physiologic pH, while no reaction was observed for secondary amines; the basis of the exceptional stability appears to be a consequence of favorable H-bond interactions of the imine nitrogen with the 5-OH group of PLP. Amphotericin B and nystatin in their native forms display marked aqueous insolubility, and possess lone primary amines. We were able to utilize PLP in achieving excellent solubilization of both these antifungal agents, surpassing aqueous solubilities of 100 mg/mL. In *in vitro* bioassays, both polyenes in their PLP-adducted form display attenuated antifungal potencies which is attributable to 'prodrug-like' complexes. These results point to the utility of excipient-free, entirely aqueous formulations of amphotericin B for parenteral use, and may also be extended to other primary amine-bearing compounds exhibiting poor aqueous solubility.

### Keywords

Amphotericin B; Nystatin; Pyridoxal phosphate; Aqueous solubilization

### Introduction

Several drugs in current clinical use display very poor aqueous solubility. Parenteral formulations of drugs classified as Class II (high permeability, low solubility), or Class IV (low permeability, low solubility) in the Biopharmaceutics Classification System<sup>1,2</sup> are often challenging, and occasionally insuperable. Although we had earlier examined the issue of solubilizing Amphotericin B (AmB) by covalent modification of the mycosamine residue,<sup>3</sup> our interest in developing general approaches toward overcoming problems with solubility of drugs perhaps began almost three decades ago when, as a medical student, one of the

Corresponding Author Address: Sunil A. David, MD, PhD, Department of Medicinal Chemistry, University of Kansas, Multidisciplinary Research Building, Room 320D, 2030 Becker Drive, Lawrence KS 66047, Tel: 785-864-1610; Fax: 785-864-1961, sdavid@ku.edu.

authors had noted but not understood at the time why, nurses in the ICU had held that dextrose solutions were better than any other commonly used fluids at ‘clearing’ intravenous lines and preventing thrombophlebitis.

Recent experiences with formulating DS-96 (Fig. 1), an *N*-alkylhomospermine lipopolysaccharide-sequestering agent<sup>4</sup> for preclinical evaluation in animal models were beset by unanticipated problems with aqueous solubility for although the polyamine compound would be expected to be very soluble, several salt forms, including the pharmaceutically acceptable hydrochloride salt, were not. We wondered whether the ‘dextrose solution’ that the ICU nurses had relied upon could be brought to bear on this problem, reasoning that the highly water-soluble aldose could reversibly react with the primary and secondary amines of the polyamine, forming transient imine and iminium species, respectively. We were pleased when we found that the compound was indeed exceptionally soluble with dextrose.

Our success, however, could not be applied to more recalcitrant drugs such as AmB and nystatin (Fig. 1). The antifungal agent AmB, and its closely related analogue nystatin are notoriously insoluble and, unlike DS-96, AmB and nystatin would not yield at all to glucose; insignificant solubility was observed even in the presence of other co-solvents. We surmised that a pharmacologically innocuous, charged, polar, aldehyde would confer auxiliary solvation that neutral glucose could not. Pyridoxal phosphate (PLP; vitamin B<sub>6</sub>) seemed eminently suitable, and indeed DS-96 was freely soluble when formulated with PLP. Both AmB and nystatin possess a lone primary amine on the mycosaminyl residue, whereas DS-96 has a single terminal primary amine and four secondary amines (Fig. 1),<sup>5</sup> and it was of interest to examine whether primary or secondary amines could interact with PLP to form imine or iminium adducts, respectively, with sufficient stability as to confer aqueous solubility. We began with spectroscopic and crystallization studies with simple, model primary and secondary amines which unequivocally showed that PLP reacts with primary, but not secondary amines, leading to the formation of stable imine adducts which are stabilized by favorable H-bond interactions with the 5-OH group of PLP. Noting, as mentioned earlier, that AmB and nystatin have a single primary amine, we asked whether the complexation with PLP would allow aqueous formulations of these otherwise insoluble antifungal agents to be obtained. We found that both AmB and nystatin are easily solubilized in water, in the presence of PLP, achieving concentrations of >100 mg/mL (the highest concentration tested). The development of aqueous-soluble formulations of AmB, in particular, may have significant clinical implications.

## Materials and Methods

Ethylamine, diethylamine, PLP, AmB, and nystatin, were procured from Sigma-Aldrich (St. Louis, MO). Multiplicity-edited <sup>1</sup>H-<sup>13</sup>C HSQC was acquired on a 500 MHz Bruker AVII spectrometer equipped with a cryogenically cooled probe. This experiment was collected with 4 transients per increment and an interscan delay of 2 s for a total acquisition time of 36 minutes. 256 and 1024 data points with spectral widths of 27672.80 and 8012.82 Hz were acquired in t<sub>1</sub> and t<sub>2</sub>, respectively. The proton and carbon carrier were set to 4.703 and 100 ppm, respectively. Crystals of 1:1 (molar equiv.) mixture of *N*<sup>1</sup>-methylpropane-1,3-diamine and PLP were obtained from a 1:1 methanol-water mixture. The structure of this model diamine-PLP complex was solved at the University of Kansas Small-Molecule X-Ray Crystallography Laboratory. All hydrogen atoms were located from a difference Fourier and included in the structural model as individual isotropic atoms whose parameters were allowed to vary in least-squares refinement cycles.

### Synthesis of pyridoxal phosphate adduct of ethylamine

The ethylamine-PLP adduct was prepared by the addition of PLP monohydrate (32.52 mg, 0.123 mmol) to ethylamine hydrochloride (5 mg, 0.61 mmol) in 2:1 molar ratio in 0.7 mL D<sub>2</sub>O. Anhydrous sodium carbonate was thereafter added to the clear solution of D<sub>2</sub>O to adjust its pH to 7. The diethylamine-PLP adduct was prepared by the addition of PLP monohydrate (24.19 mg, 0.091 mmol) to diethylamine hydrochloride (5 mg, 0.046 mmol) in 2:1 molar ratio in 1 mL formic acid. The reaction was stirred for 15 minutes and the formic acid was thereafter completely removed under reduced pressure to obtain a yellow solid, which was solubilized in 0.7 mL D<sub>2</sub>O and pH adjusted to 7 by adding anhydrous sodium carbonate to the clear solution of D<sub>2</sub>O.

### Synthesis of <sup>15</sup>N-Alanine methyl ester, and *N*-ethyl <sup>15</sup>N-alanine methyl ester

<sup>15</sup>N-alanine methyl ester (model primary amine) was synthesized by reaction of <sup>15</sup>N-alanine (Sigma-Aldrich) in methanol using 1.5 equivalents of thionyl chloride under anhydrous conditions; the reaction was stirred for 24 hours and then the solvent was removed under vacuum and dried to obtain the product in quantitative yields. <sup>15</sup>N-Ethyl alanine methylester (model secondary amine) was obtained by reductive amination of <sup>15</sup>N alanine methylester and acetaldehyde (0.7 eq.) using sodium cyanoborohydride and 4–5 drops of acetic acid. The reaction mixture was stirred for 4 hours and the solvent was then removed under vacuum to obtain the crude residue. In order to facilitate isolation by conventional flash chromatography, the crude residue was first *N*-Boc protected using (Boc)<sub>2</sub>O and triethylamine. The *N*-Boc group was then deprotected using HCl/dioxane solution by stirring for 12 hours. The solvent was then removed under vacuum and the residue was dried to obtain the <sup>15</sup>N-ethyl alanine methylester. Both the above model amines were dissolved in 85% H<sub>2</sub>O/D<sub>2</sub>O with or without 2.0 equiv. of PLP, and the pH was adjusted to 7.4 with anhydrous Na<sub>2</sub>CO<sub>3</sub>. 2D-<sup>15</sup>N- <sup>1</sup>H HSQC NMR spectra were acquired using Bruker Avance 800 MHz NMR equipped with TCI cryo probe. The spectra were recorded with 1024 complex points in <sup>1</sup>H (t<sub>2</sub>, acquisition) dimension and 32 complex points in <sup>15</sup>N (t<sub>1</sub>, indirect) dimension with sweep widths of 12820 Hz in <sup>1</sup>H and 2595 Hz in <sup>15</sup>N and 32 scans per t<sub>1</sub> increment. The sample temperature was maintained at 298 K (25° C). Identical acquisition parameters were used for all samples.

### Synthesis of pyridoxal phosphate adducts of AmB and nystatin

To a solution of 20.0 mg of AmB or nystatin in 2 mL of dimethylformamide (DMF) [10.8 mM] was added a solution of 20 mg of PLP in 2 mL of water (adjusted to pH 7.4 with Na<sub>2</sub>CO<sub>3</sub>) [40.4 mM]. Both solutions were flash-frozen in liquid nitrogen and lyophilized overnight using a VirTis AdVantage Bench Top Freeze Dryer. The resultant solids were all found to be soluble upon addition of water; both AmB- and nystatin-PLP adducts were found to be soluble at 100 mg/mL (the highest concentration tested). The presence of the polyene antifungals in aqueous solution was confirmed by LC-MS.

### LC-MS Characterization

The PLP adducts were analyzed by reverse-phase LC-ESI-MS performed on a Shimadzu LC system (LC-10AD binary pumps, SCL-10A diode array detector) using a Zorbax 3.0 mm x 150 mm 3.5 μm stable-bond C<sub>18</sub> reverse-phase column with a forty-minute binary gradient (10–100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (TFA)/0.1% TFA in H<sub>2</sub>O). ESI-MS data was acquired on an Agilent LC/MSD-TOF instrument with a mass accuracy of 3 ppm and a range of 100–3500 Daltons. Calibration drift was minimized on a scan-by-scan basis to less than 10 ppm by using internal standards corresponding to 922.0001 and 2721.0201 marker ions infused concurrently through a second nebulizer in the ionization chamber. MS acquisition was commenced 5–7 min after injection to avoid

contamination of the ionization chamber (as well as ion suppression) by excess PLP and inorganic solutes.

### Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations of reference AmB and nystatin (stock solutions at 100  $\mu\text{g/mL}$  in DMSO) and the AmB-PLP and nystatin-PLP adducts (100  $\mu\text{g/mL}$  in  $\text{H}_2\text{O}$ ) were determined by appropriate broth microdilution methods as described previously.<sup>3,6</sup> Compounds were serially two-fold diluted in quadruplicate in a 384-well microtiter plate in Sabouraud's dextrose broth using a Biotek Precision 2000 automated microplate pipetting system. A single colony of *C. albicans* ATCC 26278 was dispersed in broth and was added to the wells with appropriate vehicle-only controls. The microtiter plates were incubated overnight at 37°C and read at an absorbance of 600 nm.

## Results and Discussion

The aldehyde of PLP is well known to form imines with the  $\epsilon$ -amino group of lysine in the active site of PLP-dependent transaminases as well as with the amines of amino acid substrates.<sup>7–9</sup> Given that the otherwise aqueous insoluble DS-96 was found to be freely soluble in the presence of PLP, we were not sure if secondary amines could also interact with PLP to form quasi-stable iminium adducts. We therefore set out to examine the reactivity of primary and secondary amines with PLP under aqueous conditions using ethylamine and diethylamine as simple model amines.

In  $^1\text{H}$  NMR experiments, the addition of PLP to ethylamine (2:1 molar ratio) in  $\text{D}_2\text{O}$  (adjusted to pH 7 with  $\text{Na}_2\text{CO}_3$ ) resulted in the appearance of a new imine proton at 8.88 ppm; since an excess of PLP was used, the aldehydic proton at 10.30 ppm was, as expected, also observed (Fig. 2A). Importantly, evidence for the coexistence of complexed and free ethylamine was clearly evident by the presence of two distinct sets of the methyl and methylene proton resonances of the  $\text{EtNH}_2$  (Fig. 2A). In  $^{13}\text{C}$ - $^1\text{H}$  HSQC experiments, all resonances (other than quaternary carbons) corresponding to the expected imine adduct were observed; the imine crosspeak at 8.88 ppm in the  $^1\text{H}$  dimension corresponded to 164.94 ppm in the  $^{13}\text{C}$  spectrum (Fig. 2B). Consistent with these observations, we also observed the appearance of an imine cross-peak in  $^{15}\text{N}$ - $^1\text{H}$  HSQC experiments with  $^{15}\text{N}$ -labeled alanine methyl ester (Fig. 3). No evidence for stable adducts of PLP with either diethylamine (or *N*-ethyl  $^{15}\text{N}$ -alanine methyl ester) were, however, observed under a variety of conditions (data not shown), indicating clearly that only primary, but not secondary amines form stable adducts with PLP. We were at first surprised that the half-life of the imine adducts would be so long (relative to NMR time-scales) at physiologic pH in pure aqueous conditions as to yield distinct spectroscopic signatures, but we noted that this unexpectedly large stability could be a consequence of favorable H-bonds as had been noted earlier in crystal structures.

7

We wanted not only to explore the structural basis of the stability of the adduct, but also obtain definitive evidence for the participation of primary, but not secondary amines with PLP. A crystal structure of PLP adducted to a model diamine (*N*<sup>1</sup>-methylpropane-1,3-diamine) showed the formation of the anticipated H-bond-stabilized imine with the terminal primary amine, whilst the secondary amine (N3) was found to be free (Fig. 4).

We next asked whether the stable adducts that are formed with primary amines and PLP could be applied to clinically-relevant, more complex molecules. Adducts of the antifungals, AmB and nystatin, obtained by lyophilization of the polyene-vitamin mixtures in DMF/ $\text{H}_2\text{O}$ , were found to be highly water-soluble (>100 mg/mL). The PLP adducts display lowered antifungal potencies *in vitro*; this is attributable to the expected preponderance of

the prodrug-like adduct owing to the deliberate excess of PLP employed to increase mass-action-driven adduction (Fig. 5A). Examination of aqueous solutions of the AmB-PLP adduct by LC-MS demonstrated unequivocally the presence of AmB ( $M+Na^+$ : 946.46 amu; Fig. 5B) with its distinct absorption spectrum (Fig. 5C). Control experiments with reference AmB dissolved in DMSO showed identical retention times, absorption and mass spectra. Similar data were obtained for nystatin (data not shown).

The pharmaceutical applications of pyridoxal prodrug-like adducts are self-evident. Specifically, the toxic deoxycholate formulation of AmB can now be eliminated and replaced with purely aqueous formulations for parenteral use. These results may perhaps also be extended to other insoluble primary amine-bearing drugs.

## Acknowledgments

Tim Day is grateful for undergraduate student support by the KU Initiative for Maximizing Student Diversity (IMSD; NIH 5R25GM062232) program of the National Institute of General Medical Science (NIGMS), a component of the National Institutes of Health (NIH). He would also like to thank all of the members of the David lab whose excellent teaching and guidance made this work possible.

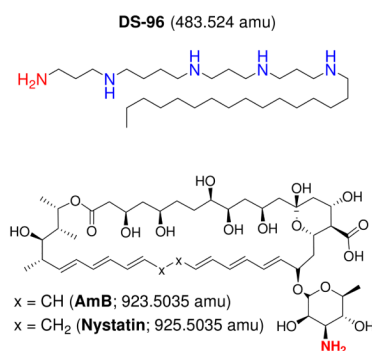
## Abbreviations

<b>AmB</b>	amphotericin B
<b>ESI</b>	electrospray ionization
<b>LC-MS</b>	liquid chromatography-mass spectrometry
<b>MIC</b>	minimum inhibitory concentration
<b>PBS</b>	phosphate-buffered saline
<b>PLP</b>	pyridoxal phosphate
<b>TFA</b>	trifluoroacetic acid

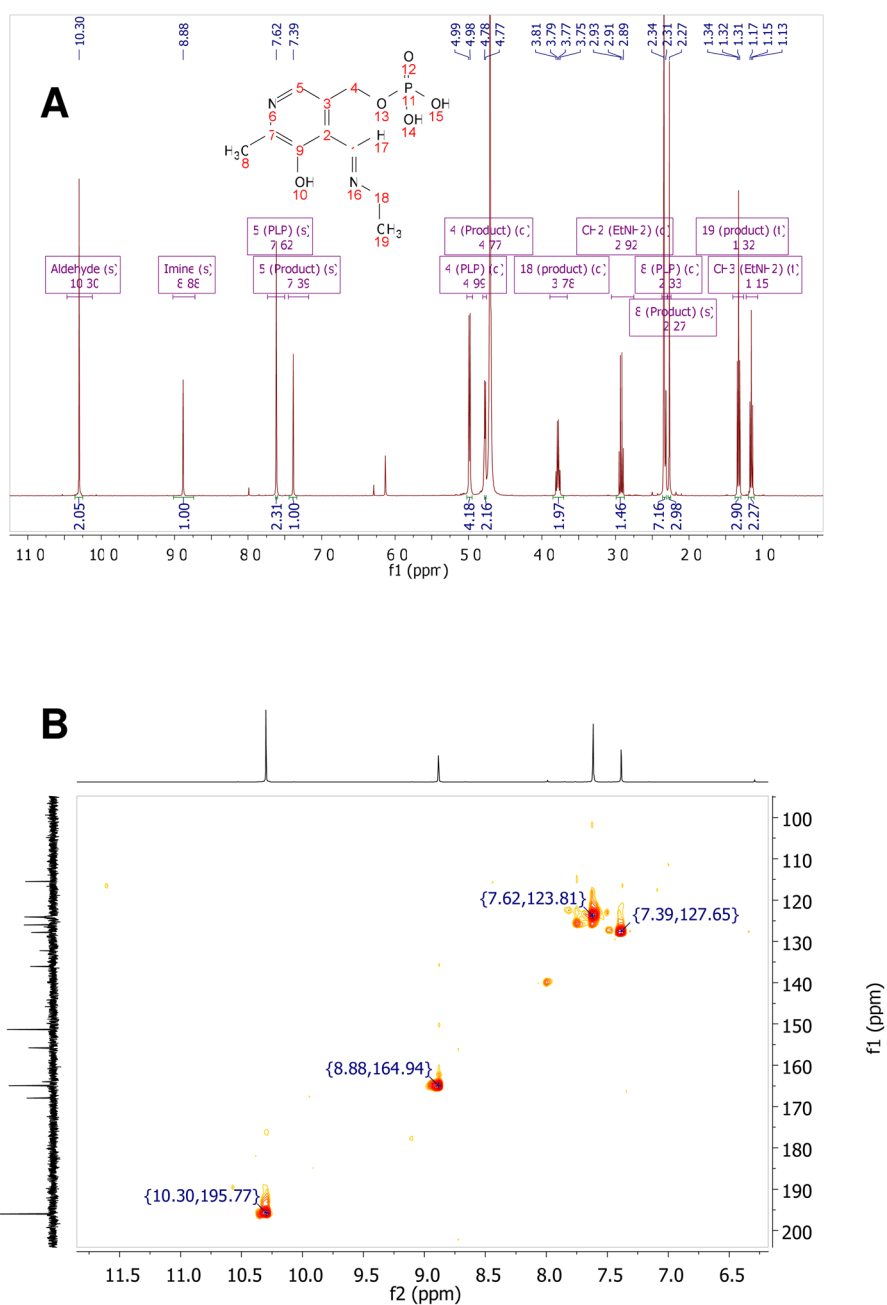
## References

1. Amidon GL, Lennernas H, Shah VP, Crison JR. A theoretical basis for a biopharmaceutical drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharm Res.* 1995; 12:413–420. [PubMed: 7617530]
2. Fagerholm U. Evaluation and suggested improvements of the Biopharmaceutics Classification System (BCS). *J Pharm Pharmacol.* 2007; 59:751–757. [PubMed: 17637167]
3. Adediran SA, Day TP, Sil D, Kimbrell MR, Warshakoon HJ, Malladi SS, David SA. Synthesis of a highly water-soluble derivative of amphotericin B with attenuated proinflammatory activity. *Mol Pharm.* 2009; 6:1582–1590. [PubMed: 19663403]
4. Sil D, Shrestha A, Kimbrell MR, Nguyen TB, Adisechan AK, Balakrishna R, Abbo BG, Malladi S, Miller KA, Short S, Cromer JR, Arora S, Datta A, David SA. Bound to Shock: Protection from Lethal Endotoxemic Shock by a Novel, Nontoxic, Alkylpolyamine Lipopolysaccharide Sequesterant. *Antimicrob Agents Chemother.* 2007; 51:2811–2819. [PubMed: 17548488]
5. Shrestha A, Li R, Sil D, Pardeshi NN, Schwarting N, Schorno KS, Rajewski RA, Datta A, David SA. Pharmacokinetics of DS-96, an alkylpolyamine lipopolysaccharide sequesterant, in rodents. *J Pharm Sci.* 2008; 97:5376–5385. [PubMed: 18383338]
6. Balakrishna R, Wood SJ, Nguyen TB, Miller KA, Suresh Kumar EV, Datta A, David SA. Structural correlates of antibacterial and membrane-permeabilizing activities in acylpolyamines. *Antimicrob Agents Chemother.* 2006; 50:852–861. [PubMed: 16495242]
7. Shen BW, Hennig M, Hohenester E, Jansonius JN, Schirmer T. Crystal structure of human recombinant ornithine aminotransferase. *J Mol Biol.* 1998; 20(277):81–102. [PubMed: 9514741]

8. Graber R, Kasper P, Malashkevich VN, Sandmeier E, Berger P, Gehring H, Jansonius JN, Christen P. Changing the reaction specificity of a pyridoxal-5'-phosphate-dependent enzyme. *Eur J Biochem.* 1995; 232:686–690. [PubMed: 7556224]
9. Sharif S, Schagen D, Toney MD, Limbach HH. Coupling of functional hydrogen bonds in pyridoxal-5'-phosphate-enzyme model systems observed by solid-state NMR spectroscopy. *J Am Chem Soc.* 2007; 129:4440–4455. [PubMed: 17371021]

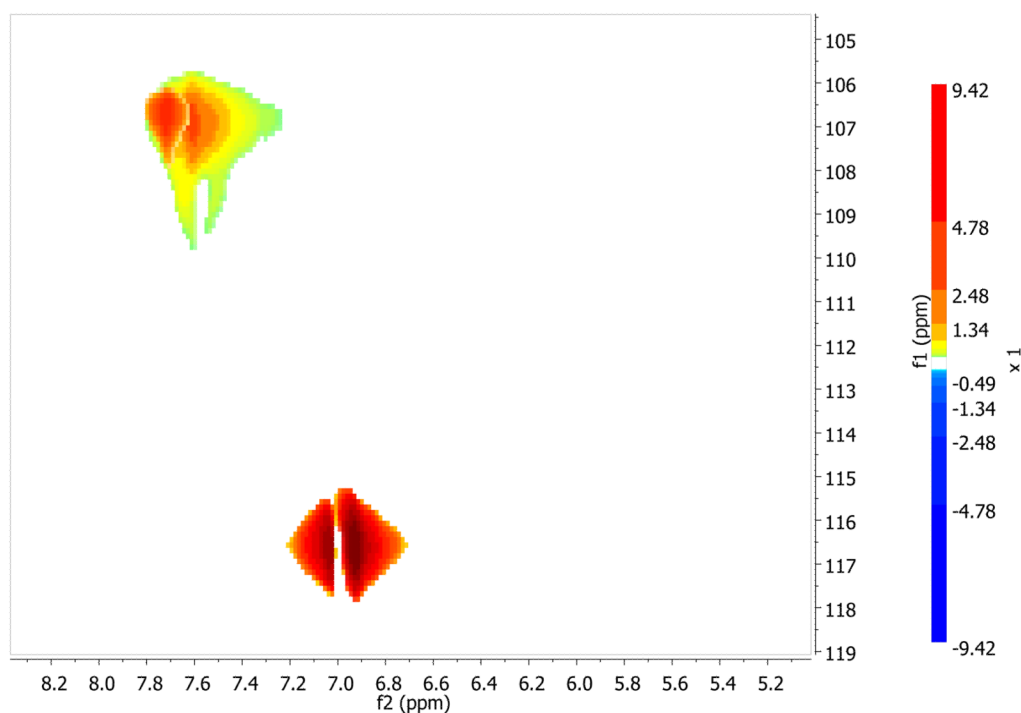


**Fig. 1.**  
Structures of DS-96, AmB, and nystatin. Primary amines are colored red, while secondary amines are in blue.

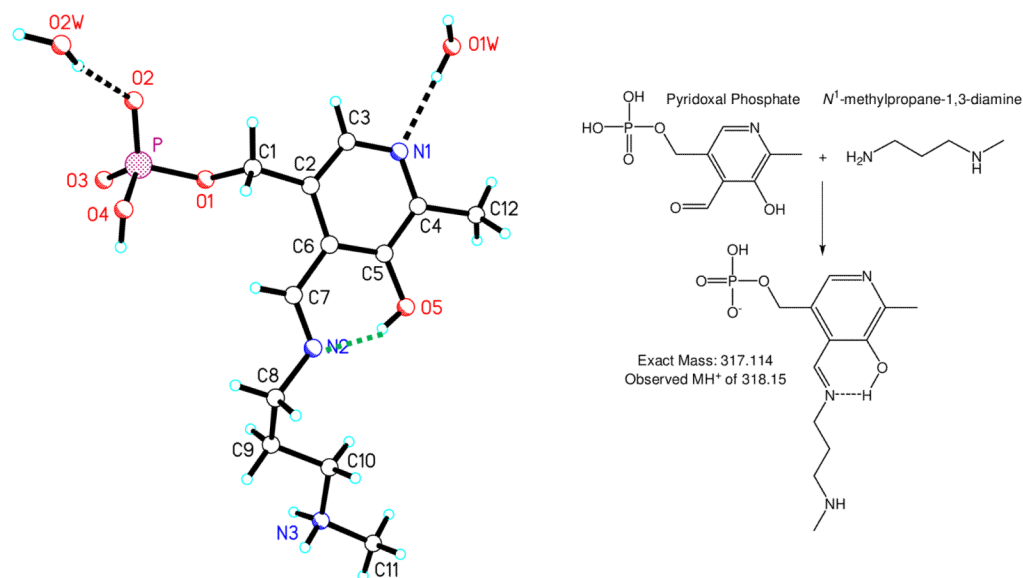
**Fig. 2.**

A.  $^1\text{H}$  NMR spectrum of a 1:2 molar ratio mixture of ethylamine and PLP in  $\text{D}_2\text{O}$ , showing the imine proton at 8.88 ppm and two distinct sets of methyl and methylene proton resonances indicating the coexistence of complexed and free amine. B. Partial  $^{13}\text{C}$ - $^1\text{H}$  HSQC NMR spectrum of ethylamine-PLP in  $\text{D}_2\text{O}$ . The imine crosspeak at 8.88 ppm ( $^1\text{H}$  dimension) and 164.94 ppm ( $^{13}\text{C}$  dimension) is clearly evident.

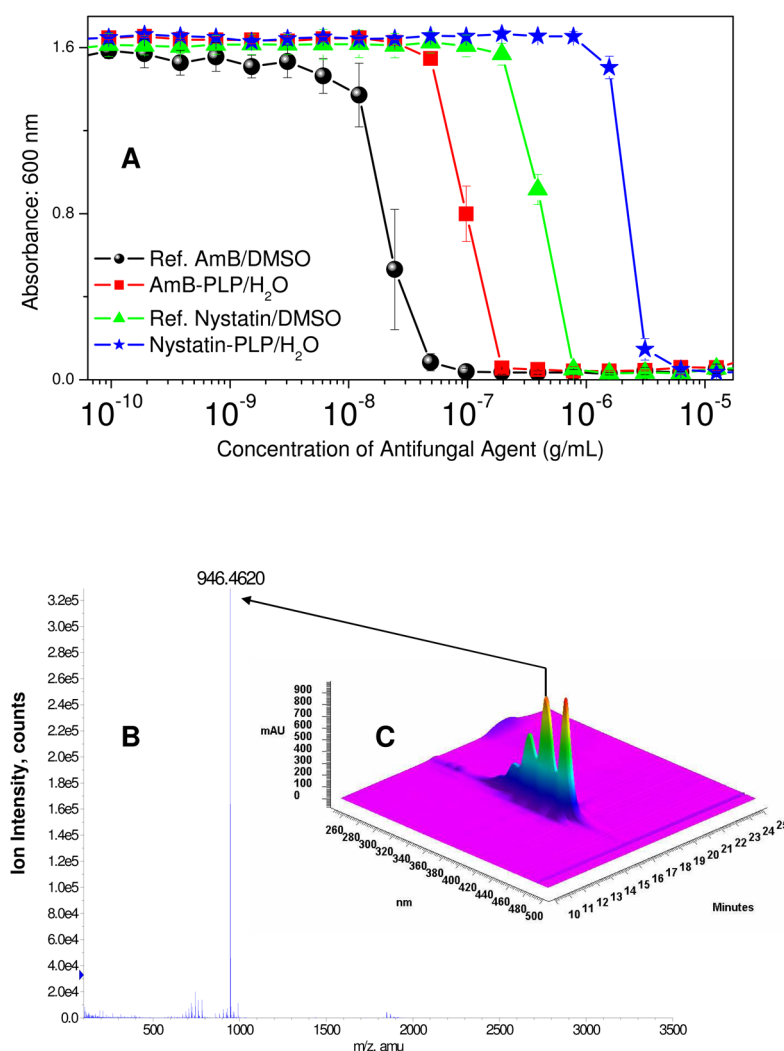


**Fig. 3.**

Overlay, of  $^{15}\text{N}$ - $^1\text{H}$  HSQC NMR of  $^{15}\text{N}$ - alanine, methyl ester and reaction of  $^{15}\text{N}$  alanine-methyl ester with pyridoxal phosphate. Samples were prepared in 85%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ . The crosspeak centered at 107 ppm ( $^{15}\text{N}$  dimension) and 7.7 ppm ( $^1\text{H}$  dimension) corresponds to the free primary amine while the crosspeak at 117 ppm ( $^{15}\text{N}$  dimension) and 7 ppm ( $^1\text{H}$  dimension) appears after the addition of PLP and is attributable to the imine.



**Fig. 4.**  
The crystal structure of *N*<sup>1</sup>-methylpropane-1,3-diamine:PLP adduct showing the participation of the primary amine, and H-bond mediated stabilization of the resultant imine.

**Fig. 5.**

A. Inhibition of growth of *C. albicans* by reference AmB and nystatin (in DMSO) and the AmB- and nystatin-PLP adducts (in water) determined by microdilution method. Data represent means and SD obtained on quadruplicates. B. LC-MS of an aqueous solution of AmB-PLP showing the expected  $M+Na^+$  ion (946.46 amu). C. Inset indicates the retention time and real-time absorption spectra (photodiode array detector output) of AmB.